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14. ABSTRACT

Our laboratory has previously shown that two isoforms of Epidermal Growth Factor Receptor family member, HER4: Cyt1 and Cyt2 exhibit opposing effects on mammary epithelial cells in vitro and in vivo. In our hands, Cyt1 attenuated growth and promoted differentiation, while Cyt2 promoted cell proliferation of mammary epithelial cells. The two isoforms differ by presence of additional 16 amino acids in Cyt1, which introduces a phosphoinositide-3 kinase- and third WW-domain binding motives; however, do not explain the different biological effects of the isoforms. We have focused our studies on a transcriptional regulator and an oncogene, Yap and have shown that Cyt1 preferentially binds Yap as compared to Cyt2. We confirmed these results in COS7 and 293T cells and (using mutational in vitro studies and mass spectrometry) identified tyrosines 341 and 394 of Yap as phosphorylation targets by HER4. We also found that HER4 cytoplasmic domain (s80) does not modulate localization of Yap in COS7 cells, although Yap promotes nuclear localization of s80 and these effects are not dependent on isoform nor kinase activity. HER4 expression also did not affect Yap cellular localization in vivo in a transgenic mouse model and human breast carcinoma. Due to technical difficulties in achieving adequate protein expression in mammary epithelial cells we have been unable to confirm interaction of Yap and HER4 in this cell type; however, are currently developing a new model to continue the studies.

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INTRODUCTION

Epidermal growth factor receptor (EGFR) family members are dysregulated in a number of epithelial cell malignancies, including breast cancer[1]. The first three members of the family: EGFR/HER1, HER2 and HER3 promote oncogenic transformation, more proliferative and aggressive cancer[1], and have been successfully targeted by breast cancer treatments. The role of the fourth member of the EGFR family, HER4, in cancer pathogenesis and progression is not as clearly established, but overall expression of HER4 in breast cancer tends to associate with more differentiated histopathological grade, slower proliferating tumors, and favorable patient survival [2-4].

In normal mammary tissue, HER4 is required for proper development and differentiation of the mammary epithelium during pregnancy and lactation [5] and deletion of HER4 in mammary epithelium of mice impairs mammary gland maturation and lactogenesis [5-6]. *In vitro*, over-expression or activation of HER4 in mammary epithelial cells, normal or cancerous, results in decreased growth, cell cycle arrest, increased apoptosis, and cell differentiation [7-9]. However, in experiments with other cell types including NIH3T3 or COS-7, HER4 over-expression promotes transformation and increased proliferation [10-11]. Additionally, few studies report a correlation between nuclear localization of HER4 in human breast cancer and worsened patient outcome [11-13]. Thus, additional studies are needed to elucidate the role and signaling mechanisms of HER4 in breast cancer which may significantly aid in development of new targeted therapies or prognostic tests.

The fact that HER4 mRNA can be alternatively spliced into four isoforms (JMa, JMb, Cyt1 and Cyt2 (Fig. 1) may be one possible reason for the conflicting data on the function of HER4 [14-15]. The JMa isoforms contains a TNF-α converting enzyme (TACE) cleavage site in its extracellular region, but which is absent in -JMb isoform. Thus, on ligand-binding, HER4-JMa is cleaved by TACE leading to shedding of the extracellular domain and undergoes subsequent intramembraneus cleavage by γ-secretase releasing an 80 kDa soluble fragment (s80) that can localize to the nucleus or mitochondria[16], a unique ability among receptor tyrosine kinases. The JMb variant lacks the TACE cleavage site and therefore remains membrane bound. Additional variation in HER4 exists within the cytoplasmic region: the Cyt1 isoform has a 16 amino acid insert absent in Cyt2. Human breast cancers express the JMa-Cyt1 and –Cyt2 isoforms[10]; however, most studies of HER4 in breast cancer do not distinguish between these different variants.

Recent studies suggest that Cyt1 and Cyt2 isoforms regulate different cellular processes. HER4 JMa-Cyt2 promotes proliferation of breast (normal and malignant) and other cell lines *in vitro*[11], whereas Cyt1, through its unique ability to bind and activate phosphoinositide-3 kinase (PI3K), regulates cell survival and chemotaxis[17-18]. Our laboratory recently compared the effect of these isoforms on growth of mammary epithelial cells and found that expression of s80-Cyt1 slowed cell proliferation and promoted lumen formation in 3D culture consistent with cell

differentiation, while cells expressing s80-Cyt2 proliferated rapidly and failed to differentiate [19]. *In vivo,* we found that induced overexpression of human s80-Cyt1 in mammary epithelium of transgenic mice resulted in decreased proliferation, premature mammary gland maturation and lactogenesis, while induction of s80-Cyt2 resulted in epithelial hyperplasia and disorganization [19]. Additionally, injection of mouse epithelial cells overexpressing Cyt1 isoform of HER4 into a mouse mammary fat pad resulted in formation of tumors that appeared more differentiated and developed slower than control tumors [20]. These findings indicate that Cyt1 and Cyt2 variants activate differentiation and proliferation, respectively; however, the downstream cellular mechanisms regulated by the two isoforms have not been identified.

The Cyt1 and Cyt2 isoforms of HER4 differ only by 16 amino acids, which introduce an overlapping PI3K- and proline-rich, WW domain (PY)- binding motifs in the Cyt1 isoform [14]. The ability of Cyt1, but not Cyt2, to bind and activate PI3K has been demonstrated and shown to regulate cell chemotaxis and survival [17-18]. New reports confirm that HER4 isoforms exhibit some differential binding preferences as Cyt1 was found to preferentially bind an E3 ligase AIP4/Itch [21], a transcriptional co-activator Yap, and another E3 ligase WWP1 [22]. Interaction of HER4 with these proteins is mediated by the PY motif of s80 and the WW domain of these proteins; thus, presence of an additional PY motif in Cyt1 may explain the biological differences observed between the two isoforms. Functionally, interactions of HER4 with these proteins could regulate HER4 stability [23], signaling (by ubiquitination) [24], as well as ability to modulate transcription [25]. Differences in the interactome of Cyt1 and Cyt2 may explain their opposing effects on cell growth.

One of established binding partners of HER4 is Yap, a protein ubiquitously expressed in mammals, including human and mouse [26-27]. Yap was initially characterized as a Yes kinase-associated protein [28] and has since been shown to be a transcriptional activator that regulates activity of several transcription factors including RunX1 and 2, p73, and Smad7[29]. However, the primary target of Yap is thought to be the family of TEF/TEAD transcription factors, shown by Zhao *et al.* to be required for Yap-dependent regulation of cell proliferation and transformation[30].

Yap is negatively regulated by the Hippo/Warts pathway[31], which relays signals from the extracellular environment into the nucleus and through Yap-mediated regulation of cell proliferation, differentiation, and apoptosis determines organ size during development[29]. Recently the Hippo pathway has also been shown to mediate the cell-cell contact inhibition[31].

Yap is now also recognized as an oncogene as A) it is encoded on human chromosome 11q22, a region often amplified in human cancers, as well as mouse models of liver[32] and breast cancer[33], B) over-expression of Yap in the non-transformed mammary epithelial cell line (MCF10A) increases cell migration, epithelial-to-mesenchymal transition, growth factor-independent proliferation, and anchorage independent growth[33], and C) conditional over-expression of Yap in mouse liver leads to hepatomegaly and development of liver tumors[34].

BODY

Published data establish that HER4 and Yap are binding partners and our preliminary data indicate that HER4 Cyt1 exhibits enhanced ability to bind Yap as compared to Cyt2. We hypothesized that HER4 isoforms may distinctly regulate Yap activity through their differential ability to interact with Yap, which may explain their opposing effects on mammary epithelial cells. We thus designed this study to further compare the interaction of HER4 isoforms, Cyt1 and Cyt2 with Yap (Aim 1), and evaluate the effects of this interaction on Yap localization *in vitro* and *in vivo* (Aim 2) and function (Aim 3). We expected to complete majority of aims 1 and 2 during the first year of the fellowship and here summarize the research progress during this time period.

Aim 1: To characterize the interaction of HER4 s80 –Cyt1 and –Cyt2 with Yap. We will confirm the differential ability of HER4 Cyt1 and Cyt2 isoforms to phosphorylate Yap in mammary epithelial cell lines. Additionally, we will determine which Yap tyrosine(s) are targeted for phosphorylation by the HER4 isoforms.

Subtask 1a: Generation of Yap mutants.

Using Mutagenesis Kit (Pierce) and manufacturer's software to design primers, we generated four constructs encoding HA-tagged Yap1 harboring Tyr-to-Phe substitution for each of the four tyrosines present in Yap1 (Fig. 2). We introduced these mutations into Yap1 instead of Yap2, as originally proposed, due to the fact that Yap1 is the predominant isoform expressed in human [26]. We also realized that for mapping of the phosphorylation site, it will be adequate to generate mutants with single mutations, instead of combinations of mutated tyrosines, which will also decrease the possibility of any effects on protein structure due to multiple amino acid substitutions. To simplify annotation, the generated constructs were designated Y1F, Y2F, Y3F and Y4F, where each abbreviation corresponds to Y188F, Y341F, Y357F, and Y394F, respectively.

Subtask 1b. Identification of Yap tyrosine(s) targeted by s80-Cyt1 and –Cyt2 for phosphorylation in COS7 and 293T cells.

Our preliminary data indicated that HER4 binds Yap and that Cyt1 isoform binds Yap with higher affinity, as compared to Cyt2, likely contributing to enhanced phosphorylation of Yap (Fig. 3a). We confirmed these findings in two different cell lines, COS7 and 293T cells and identified the Yap tyrosine targeted for phosphorylation.

To accomplish this task, HA-tagged Yap1 or the Tyr-mutants were transiently co-expressed with Flag-tagged HER4-s80-Cyt1, -Cyt2 or kinase dead (KD) mutants in COS7 or 293T cells using Fugene (Roche) and following manufacturer's protocol. Cells were lysed 48h after transfection and immunoblotting analysis performed either directly on cell lysate or on immunoprecipitated fraction, as indicated.

We first evaluated the ability of other EGFR family members to bind Yap and found that only HER4 co-immunoprecipitates with Yap (Fig. 3b). In further experiments, we confirmed the preliminary findings that in COS7 and 293T cells Cyt1 preferentially binds Yap as compared to Cyt2 (Fig. 3c and d). Our data also indicate that HER4 interaction with Yap does not require kinase activity (Fig.3c and d). Subsequently, using the Yap1 mutants generated in Subtask 1a, we determined that HER4 targets the last tyrosine within the transactivation domain of Yap1 for phosphorylation (Y394, Fig. 4a). Additionally, these finding were confirmed by mass spectrometry (MS) with help of the University of North Carolina Michael Hooker Proteomics Center (Fig. 4b and Table 1). The MS studies also indicated high probability of HER4 phosphorylating tyrosine 341 of Yap1, which was confirmed on manual inspection of the spectrum. However, this was not detected by immunoblot when HER4 and Yap1-Y2F were co-expressed (Fig. 4a and c) suggesting that phosphorylation of Y341 maybe be dependent on and secondary to Y394 phosphorylation. Thus in Yap1 lacking Y394, both phosphorylation sites would be affected and no phosphorylation would be detected, while in Y341 mutation, only one site of phosphorylation would be lost, which might not be discernable on an immunoblot. Additionally, in some experiments we observed a decrease in tyrosine phosphorylation of Yap when tyrosine 188 was mutated (compare Fig. 4c vs. 4a); however, we attribute this finding to loss of binding between HER4 s80 and Yap which is known to be mediated by the WW domain of Yap and is dependent on the tyrosine [27]. Correspondingly, we observed less or no HER4-s80 co-immunoprecipitate with Yap1 Y1F (Fig. 4a and c). Subsequently, we compared whether HER4 isoforms target the same tyrosine(s) of Yap1 for phosphorylation and indeed found that both Cyt1 and Cyt2 phosphorylate the same tyrosine(s) (Fig. 4c).

Subtask 1c. Confirmation of s80-Cyt1 and –Cyt2 binding with and phosphorylation of Yap in mammary epithelial cells (HC11 and MCF7).

To assess whether HER4 binds Yap in mammary epithelial cells, we first co-expressed HA-Yap and HER4-s80-Cyt1, -Cyt2 or KD in HC11 cells using a Fugene protocol (Roche). However, we found that in contrast to transfections in COS7 and 293T cells, the expression level of Yap and HER4-s80 was quite low. We optimized transfection protocol using electroporation with the Amaxa system and were able to achieve higher levels of expression (Fig. 5a). Additionally, sonication of cells in lysis buffer increased solubility of the proteins and improved detection. With these technical modifications, we found detectable levels of expression (although still lower than in COS7 or 293T cells) but were able to detect weak HER4-s80 interaction with Yap in some of these samples (Fig. 5a). However the results were difficult to replicate. In our attempts to improve transfection efficiency, we employed a modified reverse transfection method commonly used to introduce siRNA into live cells on screening arrays [35]. This method also depends on Fugene or other lipid-moiety to deliver nucleic acid to the cells; however, the DNA:lipid complex is added to either partially or completely trypsinized cells, which induces cell endocytosis and promotes nucleic acid uptake by the cell resulting in increased expression levels. Indeed, we did observe higher expression levels of transfected constructs; however, did

not detect Yap: HER4-s80 interaction nor tyrosine phosphorylation of Yap (Fig. 5b). We also generated HC11 cells stably expressing, after a retroviral infection and selection, GFP (control) or GFP-tagged HER4-s80-Cyt1 or –Cyt2. We interrogated these cells for HER4-s80 interaction with endogenous Yap and tyrosine phosphorylation. Again, we were able to detect some interaction between the two proteins, but were able to detect tyrosine phosphorylation of Yap, and even s80, only after 5 min treatment of cells with pervanadate, a potent and promiscuous inhibitor of all tyrosine phosphatases (Fig. 5c).

We have found similar difficulties in investigating Yap and HER4-s80 interaction in MCF7 cells. Initial attempts at transient transfection yielded low levels of expression; however, were improved greatly by employing the reverse transfection protocol (Fig. 6). However, despite good expression levels of both Yap and HER4-s80, we have been unable to detect any interaction between these two proteins, nor tyrosine phosphorylation of Yap.

These results indicate that HER4-s80 interaction with Yap may be cell type specific and does not occur in mammary epithelial cells or is prevented due to compartmentalization or regulatory mechanism(s) that are able to compensate for protein overexpression. COS7 and 293T cells either lack these mechanisms due to cell immortalization/transformation or are not able to compensate very high expression of both Yap and HER4-s80, leading to artificial protein interactions. It is important to note, that in mammary epithelial cells *in vivo*, HER4 expression is maintained at really low levels. Alternatively, the HER4:s80 interaction might be only occurring in the nucleus of the mammary epithelial cells and the interacting proteins are retained within the insoluble fraction during cell lysis. We examined Yap and HER4-s80 cellular localization using cellular fractionation kit (Pierce) and could not detect these proteins in the nucleus. Requirement of the nuclear fractionation method for high salt buffer prevented us from performing co-immunoprecipitation experiments. Lastly, sonication, which greatly improves protein detection in HC11 and MCF7 cell lysates, may be causing disruption of the protein complexes and nonspecific protein aggregation[36] and thus prevents detection of HER4-s80:Yap interaction. We have tested different lysis buffers, but were unable to detect transfected proteins as well as when sonication was used.

We are currently developing MCF7 cells that express HER4-s80-Cyt1, -Cyt2 or KD under control of doxycycline-inducible promoter (RetroX retroviral TetON system from Clonetech) that might help us overcome some of the transfection/expression problems. We initially attempted to develop these cells using the tetracycline-inducible TRex system (Invitrogen); however found that due to low transfection efficiency of MCF7 cells and high sensitivity of these cells to selection agents, this approach was not feasible. Retroviral infection of MCF7 cells has so far been very effective and we are in process of selecting the cells for expression of the secondary construct (HER4-s80). MCF7 TetOn cells will allow us to achieve higher levels of HER4-s80 expression in these cells and might allow us to examine the interaction between s80 and endogenous Yap. If we are able to confirm that HER4-s80 binds and phosphorylates Yap in these cells, we will attempt to confirm the Yap tyrosine targeted by HER4 for phosphorylation.

Aim 2. To determine whether HER4 isoforms differentially regulate Yap localization. We will explore the role of HER4 s80 as a potential Yap nuclear transporter and compare the ability of Cyt1 and Cyt2 to regulate Yap localization in three different models of breast cancer.

Subtask 2a. Evaluation of cellular localization of s80-Cyt1, -Cyt2 and Yap.

We have completed the analysis of Yap and HER4-s80 localization in COS7 cells and found that Yap influences the localization of HER4 cytoplasmic domain (Fig. 6). To complete this analysis, COS7 cells were co-transfected with HA-tagged Yap and Flag-tagged HER4-s80-Cyt1, -Cyt2 or KD and 24h later plated on chamber slides. After 24h to allow for cell adhesion, cells were fixed and stained using anti-HA and anti-Flag antibodies coupled to Alexa-647 or -488, respectively, and after mounting with DAPI containing media (to counterstain the nuclei), analyzed by confocal microscopy with assistance of the Microscopy Services Laboratory at the University of North Carolina. Our data indicate that Yap expressed alone distributes throughout the cell, both in the cytoplasm and the nucleus, with some focal localization in the cytoplasm (Fig. 6, Panel 2, C). Both HER4-s80 isoforms also distributed throughout the cell, both in the cytoplasm and nucleus (Fig. 6, Panel 1, F, J, N, R). We did not observe any differences in localization of Cyt1 and Cyt2 isoforms, nor the KD mutants, which indicates that HER4 does not depend on its kinase activity for nuclear localization. These findings are in direct contrast with published data from our laboratory [16] and might be due to the fact that we employed COS7 cells for these analyses, while the published data was generated in HC11 cells. As discussed above, HER4 localization and signaling may be regulated in cell-specific manner. Alternatively, gross overexpression of HER4 in COS7 cells could result in protein accumulation in the nucleus.

However, when Yap and HER4-s80 were co-expressed, s80 localized to the nucleus in very distinct focal regions and co-localized with Yap within those regions (Fig.6E-T, Panel 2). Interestingly, Yap localization did not change significantly, and Yap was still observed in both cytoplasm and nucleus, although exhibited more focal localization within the nucleus (Fig.6, Panel 2, C vs. G, K, O, S). These data indicate that Yap localization to the nucleus is independent of HER4 cytoplasmic domain, but that Yap promotes s80 localization to the nucleus and to specific focal regions. These results further confirm that HER4-s80 bind Yap in COS7 cells, as reported above in Subtask 1b.

Due to difficulties with transfection in mammary epithelial cells (described above), these findings have not been confirmed in mammary epithelial cells. Once we develop the MCF7 TetOn HER4-s80 cell lines, that stably express Flag-tagged HER4-s80 isoforms under control of doxycycline-responsive promoter, we plan to complete the co-localization studies.

Subtask 2b: Characterization of the effect of s80-Cyt1 and –Cyt2 expression on Yap localization in mouse mammary glands.

To evaluate Yap localization in mammary glands of transgenic mice, we stained paraffin-embedded mammary glands with anti-Yap antibody (Cell Signaling) following an established

immunohistochemistry protocol. We performed the analysis on all available samples that were generated previously in our lab (see Muraoka-Cook *et al.* 2009) and approved under IACUC protocol 06-178 on 06/15/2006.

In our analysis we included mammary glands from WT mice (controls) and transgenic mice expressing s80-Cyt1, s80-Cyt2, or degradation-resistant Cyt1 (db) that were exposed to doxycycline (to induce transgene expression) for either 4 days or one year (Table 2).

Yap was found to localize to both cytoplasm and the nucleus of mammary epithelial cells in the control mice (Fig. 7). We did not observe any differences in Yap localization between tissue from WT, $Cyt1^{+}/db^{+}$ or $Cyt2^{+}$ mice, which corresponds to our findings from the *in vitro* localization experiments reported above. Differences in staining intensity are due to batch differences.

Subtask 2c. Evaluation of the relationship between HER4 and Yap nuclear localization in estrogen receptor positive human breast cancer samples.

To date we have performed a pilot study on 10 estrogen receptor positive human breast carcinomas and matching normal controls. The samples were stained for HER4 and Yap using established immunohistochemistry protocol. Within the normal samples examined, we observed HER4 expression in mammary epithelium as well as weaker staining in the stroma. HER4 predominantly localized to the cytoplasm in the normal mammary epithelium, with a few cells exhibiting nuclear localization (Fig. 8A-C). In the carcinoma tissue, we observed slight decrease in intensity of cytoplasmic staining for HER4 in nine of ten samples, but only two samples with increased nuclear stain (Fig. 8D-F).

Yap was observed only in the mammary epithelium in samples obtained from normal tissue, where it localized primarily in the cytoplasm (Fig. 8G-I). In the carcinoma, Yap cytoplasmic expression decreased in 7 out of ten samples, however there were no changes in nuclear expression in those samples (Fig. 8J-L). We observed increase in Yap cytoplasmic expression in only one carcinoma sample as compared to its matching control.

In eight samples out of ten tested we observed changes in both HER4 and Yap expression; however there was no particular trend or directionality that would indicate any relationship in localization or expression of the two proteins. As we have been unable to detect any trends in Yap or HER4 localization in the carcinoma samples as compared to normal matching tissue, we will halt further analysis to preserve precious patient samples.

Aim 3: To evaluate the functional consequences of HER4 isoform interaction with Yap. We will investigate whether binding and phosphorylation of Yap by HER4 isoforms modulate the ability of Yap to regulate TEF/TEAD-, RunX2-, and p73-dependent transcription. We will also examine whether HER4 s80–Cyt1 and –Cyt2 interact with the Yap:transcription factor complex,

specifically Yap:TEF/TEAD, and will evaluate the ability of the HER4 isoforms to directly bind and phosphorylate TEF/TEAD.

Subtasks proposed to complete Aim 3 are currently being completed.

KEY RESEARCH ACCOMPLISHMENTS:

- Generated constructs encoding HA-tagged human Yap1 (wild type) and Yap1 harboring Tyr-to-Phe mutations for each of its tyrosines (Y188F, Y341F, Y357F, and Y394F)
- Confirmed preliminary data in COS7 and 293Tcells supporting our findings that Yap is preferentially bound by HER4-s80-Cyt1 as compared to –Cyt2
- Identified Tyr 341 and Tyr394 as Yap1 tyrosines that are phosphorylated by HER4-s80 (both Cyt1 and Cyt2), with the Tyr394 being the major target
- Found that HER4-s80 either does not bind and phosphorylate Yap in mammary epithelial cells, or the interaction is subject to very stringent regulation
- Found that Yap promotes nuclear and focal localization of both HER4-s80 isoforms, which is independent of HER4 kinase activity and that HER4-s80 co-localizes with Yap. However, HER4-s80 does not modulate Yap cellular localization.
- Found that HER4-s80 does not affect Yap cellular localization in murine mammary gland *in vivo*.
- Did not find any relationship between HER4 and Yap expression and localization in human breast carcinoma as compared to normal tissue.

REPORTABLE OUTCOMES:

Misior A, Earp HS. Mediators of HER4 Cyt1 and Cyt2 isoform signaling: role of WW domain proteins. Postdoctoral Research Symposium. Abstract for an oral presentation. University of North Carolina at Chapel Hill, Chapel Hill, NC. October 2009.

Completed Cancer Pathology course (PATH725) offered by the UNC Cancer Cell Biology Training Program in May 2010

Misior A, Feng S, Earp HS. HER4 isoforms Cyt1 and Cyt2 differentially interact with Hippo pathway effectors Yap and TEAD. Postdoctoral Research Symposium. Abstract for a poster. University of North Carolina at Chapel Hill, Chapel Hill, NC. October 2010.

Misior A, Feng S, Hashmonay G, Earp HS. HER4 isoforms Cyt1 and Cyt2 differentially interact with Hippo pathway effector Yap. Abstract for a poster. Department of Defense Era of Hope Conference, Orlando, FL. August 2-5, 2011.

CONCLUSION

The data collected over the past year indicate that HER4 is the only EGFR family receptor that binds Yap and that, in COS7 and 293T cells, the cytoplasmic domain of HER4, binds and phosphorylates Yap. Cyt1 isoform preferentially binds Yap, as compared to Cyt2 and both Cyt1 and Cyt2, phosphorylate Yap on Tyr341 and Tyr394, localized within the transactivation domain of Yap1.

This is the first report of Yap phosphorylation by HER4. Previously, Yap has been shown to be phosphorylated by c-Abl, which targets Tyr357, also localized within the transactivation domain of Yap (in our notation, annotated as Y3F, see Fig. 2)[37]. This modification increased stability of Yap, promoted its binding with transcriptional factor p73, and switched p73-driven gene expression from growth arrest to proapoptotic genes. In another report, Zaidi *et al.* show that Yap is phosphorylated by Src, although the site of phosphorylation was not identified, which promoted binding of Yap with transcriptional factor RunX2, and inhibited expression of RunX2-dependent genes [38]. In both of these instances tyrosine phosphorylation of Yap modulated gene expression of Yap-regulated transcription factors; thus, it is likely that HER4-mediated tyrosine phosphorylation of Yap also results in modulation of gene expression. We are currently exploring the functional effect of the Tyr394 phosphorylation as originally proposed in Aim 3.

Unfortunately we have been unable so far to conclusively confirm that HER4 and Yap interact in mammary epithelial cells despite achieving adequate expression of both proteins. It is possible that HER4 and Yap interaction is cell-type specific and localization of HER4 and/or Yap might be highly regulated/compartmentalized in mammary epithelial cells. HER4 is required for differentiation of mammary epithelial cells[5], thus it may be highly regulated in these cells. Additionally, the two models we selected for the studies, the HC11 and MCF7 cells, despite being cell lines still maintain “normal” mammary cell biology, evidenced by their ability to differentiate when exposed to lactogenic hormones (HC11 cells)[39] and expression of estrogen receptor (MCF7 cells)[40]. Since we have been unable to detect HER4:Yap interaction on co-immunoprecipitation experiments, the best way to evaluate localization of these proteins in mammary epithelial cells will be through confocal microscopy. These studies had been already proposed under task 2a, but have been delayed due to difficulties in achieving adequate protein expression from transfection. Now that we have optimized the transfection protocols, we will evaluate cellular localization of HER4 and Yap in mammary epithelial cells. These studies will not conclusively confirm direct binding of HER4 and Yap, but will provide evidence for protein co-localization, if any. Additionally, we are currently generating MCF7 cell line that expresses Flag-tagged HER4-s80 isoforms under control of doxycycline-responsive promoter (Retro-X TetOn system from Clonetech), which we hope will help eliminate any detrimental effects long-term expression of HER4 isoforms might have on cell proliferation and survival.

We also found some limited data that tyrosine phosphorylation of Yap is enhanced in HER4-s80-Cyt1 expressing mammary epithelial cells. However, we were only able to detect the

phosphorylation when cells were pretreated with pervanadate, a potent inhibitor of tyrosine phosphatases. Interestingly, we could detect HER4-s80 tyrosine phosphorylation also only after block of phosphatase activity, while in other cells studied (COS7 and 293T) the phosphorylation was readily detected. These results provide further evidence for strict regulation of HER4-s80 in mammary epithelial cells.

Studies conducted in aim 2 indicate that HER4-s80 isoforms do not modulate Yap localization in COS7 cells; rather, Yap drives nuclear localization of HER4 to specific foci. The distribution of HER4-s80 did not differ between the Cyt1 and Cyt2 isoforms and was not dependent on kinase activity. These findings are in direct contrast to published findings from our laboratory[16], where nuclear localization of HER4-s80 was shown to require active kinase. The published studies were conducted in HC11 mammary epithelial cells, whereas our current studies were carried out in COS7 cells, and might be another evidence for cell-type specific regulation of HER4 signaling. As mentioned above, we have not carried out the localization studies in mammary epithelial cells due to insufficient expression of HER4-s80 and Yap; however, we have now optimized the transfection protocols, and, as discussed above, are generating MCF7 TetOn HER4-s80 cell lines that should allow us to complete these experiments.

We also assessed the effect of HER4 isoform expression on endogenous Yap localization *in vivo*, utilizing a transgenic mouse model expressing human HER4-s80-Cyt1 or –Cyt2 in the mammary gland under control of doxycycline-responsive promoter[41]. We have analyzed all samples available, generated in previous studies in our laboratory (no new mice were generated for this study), and found no differences in Yap cellular localization regardless of transgene expressed (Cyt1/Cyt1^{db} or Cyt2).

Similarly, we were unable to find any relationship between HER4 and Yap expression in human ER^{+} breast carcinoma samples. Published data do not agree on expression and localization of HER4 in breast carcinoma: some find less than 50%, while others report that 70% of breast cancers express HER4[42]. In the most recent and most comprehensive analysis of HER4 expression in invasive breast carcinoma, Thor *et al.*[13] analyzed 923 samples and found HER4 expression in 68% patients. The authors also reported that HER4 localized to the cytoplasm in 63% of samples, to the nucleus in 23%, and to both in 18%. In our limited analysis, we predominantly observed HER4 positivity in cytoplasm and only few cells exhibited nuclear localization. We did not observe significant differences in HER4 localization between normal tissue and carcinoma samples.

Yap has been reported primarily in mammary epithelial cells and myoepithelial cells, both in cytoplasmic and nuclear compartments[43] and we observed similar localization. Similarly, we did not observe any changes in Yap localization in breast carcinoma as compared to normal tissue[43]. To date we completed the analysis in a pilot study of ten samples and considering lack of significant differences, we will not continue the analysis to preserve limited patient samples.

We are currently continuing with studies proposed under Aim3 that evaluate the effect of HER4-dependent tyrosine Yap phosphorylation on Yap function and will complete the analysis of Yap and HER4 localization in mammary epithelial cells by confocal microscopy. Even though our data has not confirmed our hypothesis to date, the findings provide valuable information about HER4-dependent signaling as well as to cell-type specific HER4 regulation. We are also exploring proteomic and genetic approaches to studying the differences in HER4 isoform-specific signaling in mammary epithelial cells, which will increase our understanding of HER4 regulation and function and will contribute to our knowledge about processes that control carcinogenesis in the breast.

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SUPPORTING DATA

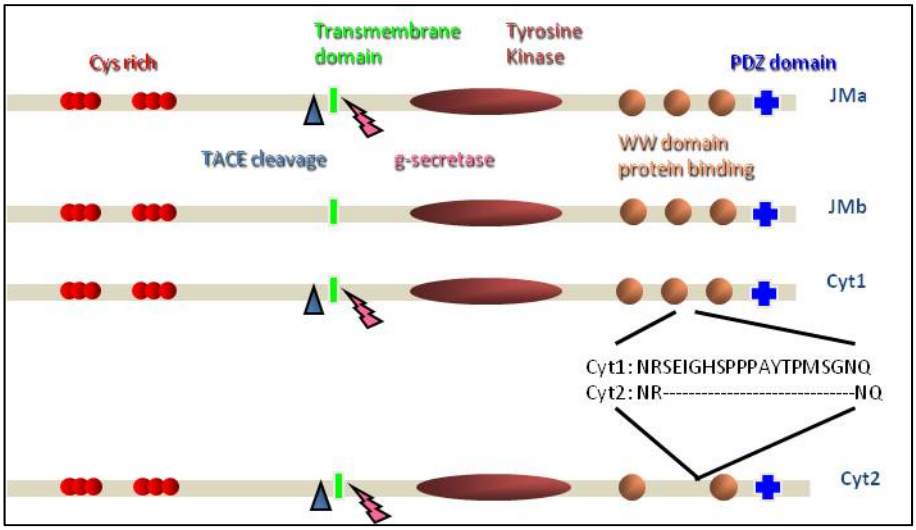


Figure 1. HER4 isoforms. Due to alternative mRNA splicing, HER4 exists as four isoforms: JMa contains a TACE cleavage site in the transmembrane region, which is absent in JMb, while Cyt1 contains additional 16 amino acids within its cytoplasmic domain. The TACE cleavage site allows for JMa to be cleaved by TACE and subsequently be processed by γ-secretase to release an 80 kDa cytoplasmic domain (s80), which has been shown to translocate to the nucleus and mitochondria. Extra 16 amino acids present in Cyt1 contain a PI3K and WW domain binding motives.

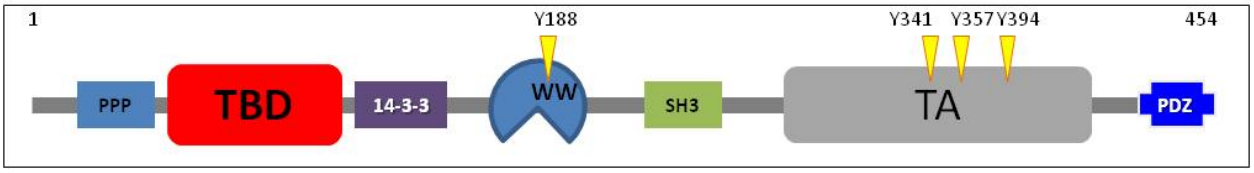


Figure 2. Domain structure of Yap. Diagram depicting domain structure of predominant human Yap isoform (Yap1). Yap contains a proline rich region (PPP), TEF/TEAD binding domain (TBD), 14-3-3 protein binding motif (14-3-3), WW domain (WW), SH3 binding motif (SH3), transactivation domain (TA), and a C-terminal PDZ domain (PDZ). Arrowheads indicate location of tyrosine residues in relation to the functional domains of Yap.

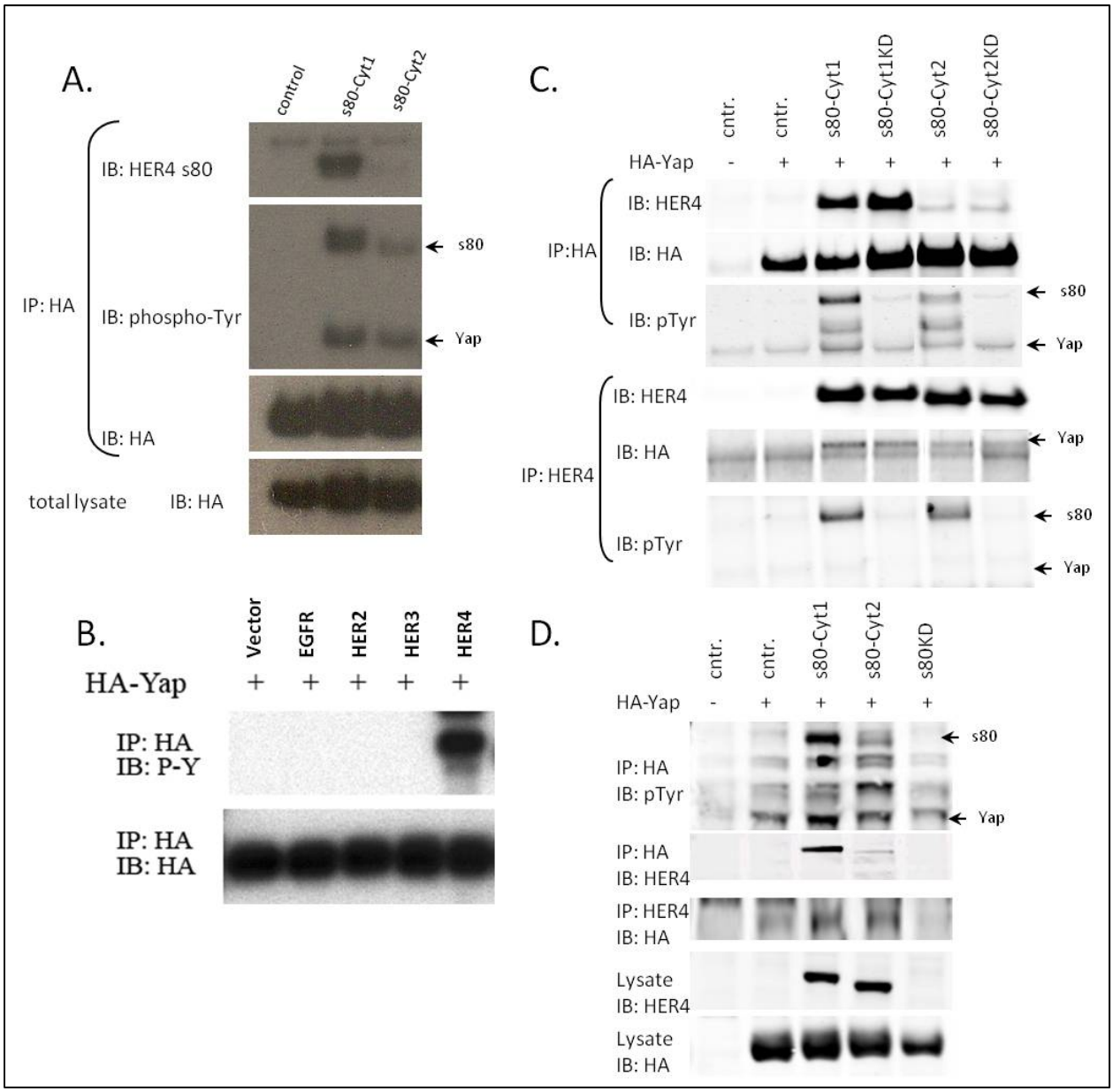


Figure 3. Yap preferentially binds HER4-s80-Cyt1. A. Preliminary data indicating that Yap preferentially binds HER4-s80-Cyt1, as compared to –Cyt2. **B.** Yap binding with EGFR family members. EGFR family members were co-expressed with HA-Yap in COS7 cells and 48h later lysates interrogated for receptor-Yap interaction. **C and D.** HER4-s80 interaction with Yap in COS7 (C) and 293T (D) cells. HA-Yap was co-expressed with s80-Cyt1, -Cyt2 or kinase dead (KD) mutants. 48h after transfection cells were lysed and lysates immunoprecipitated and analyzed by immunoblot as indicated.

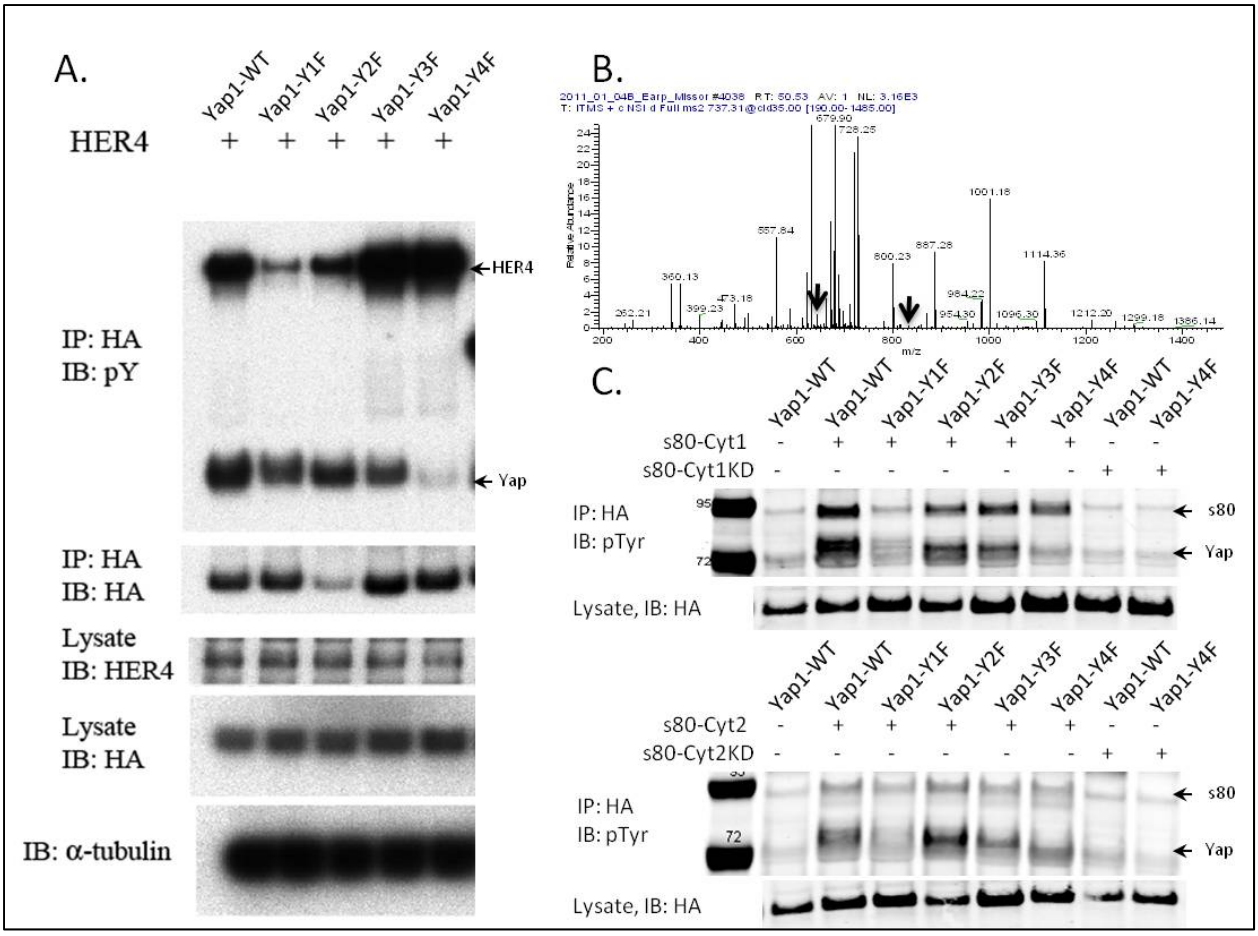


Figure 4. HER4 phosphorylates Yap1 on Y341 and Y394. A. HER4-s80-Cyt1 was co-expressed with HA-Yap WT or indicated mutants in COS7 cells. 48h after transfection lysates were immunoprecipitated as indicated and analyzed by immunoblotting. **B.** Mass spectrum of peptide fragment containing Y394 of Yap1 for manual confirmation of phosphorylation. HA-Yap (WT) was co-expressed with HER4-s80-Cyt1 in COS7 cells. 48h later cells were lysed, lysate immunoprecipitated with anti-HA affinity gel, and run on polyacrylamide gel. Band corresponding to HA-Yap (~72kDa), was excised and digested with AspN, then analyzed on LTQ Orbitrap mass spectrometer. A total of 11 spectra matched to peptides containing Y394 and two were potentially phosphorylated. Manual verification of these peptides supports the phosphorylation of Y394. Arrows indicate peaks used to distinguish pTyr vs. pThr. Study completed with help from the University of North Carolina Proteomics Core Facility. **C.** Comparison of Yap phosphorylation by Cyt1 and Cyt2. COS7 cells were co-transfected with control vector (pcDNA3.1), s80-Cyt1, -Cyt2 or kinase dead variants and either wild type or mutated HA-tagged Yap. 48h after transfection, cells were lysed, lysates immunoprecipitated with anti-HA affinity gel, and analyzed by immunoblotting as indicated.

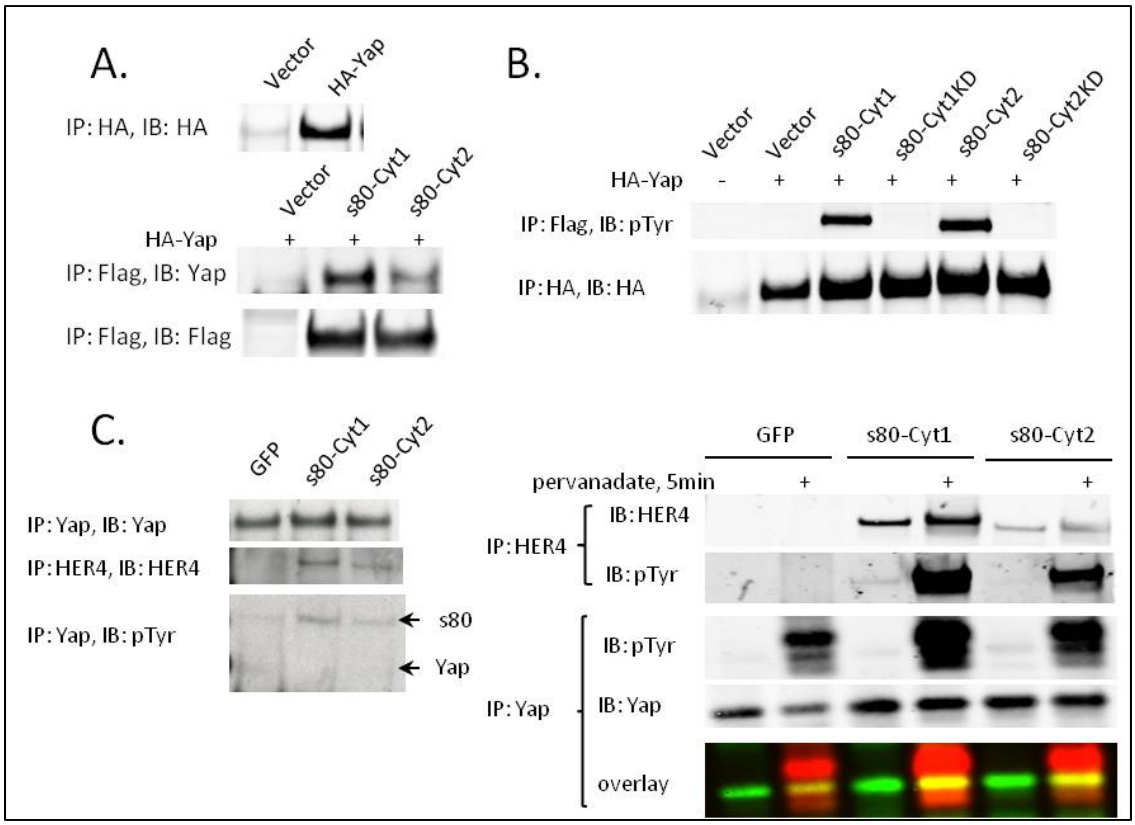


Figure 5. Interaction of HER4-s80 and Yap in mouse mammary epithelial cells, HC11. A. Levels of HER4 isoforms and Yap 48h after electroporation with Amaxa system. Yap binding to HER4-s80 was detected, however not tyrosine phosphorylation of Yap. B. Levels of HER4 isoforms and Yap 48h after reverse transfection. No HER4:Yap interaction, nor Yap tyrosine phosphorylation were detected. C. Levels of HER4-s80 expression and endogenous Yap in HC11 cells stably expressing GFP, GFP-s80Cyt1 or GFP-s80Cyt2 (after retroviral infection). Left panel: Cyt1 and Cyt2 were detected on immunoblots of cell lysates immunoprecipitated with anti-Yap antibody. Right panel: cells were treated for 5 min prior to harvest with vehicle or pervanadate. Tyrosine phosphorylation of Yap and HER4-s80 is only detected in presence of pervanadate. Overlay panel shows co-localization of the pTyr and total Yap signals (detected by Li-Cor detection system (Odyssey)).

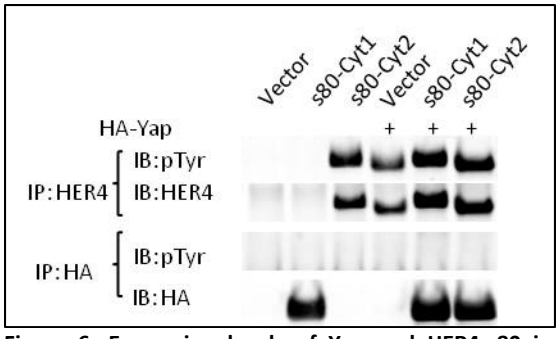
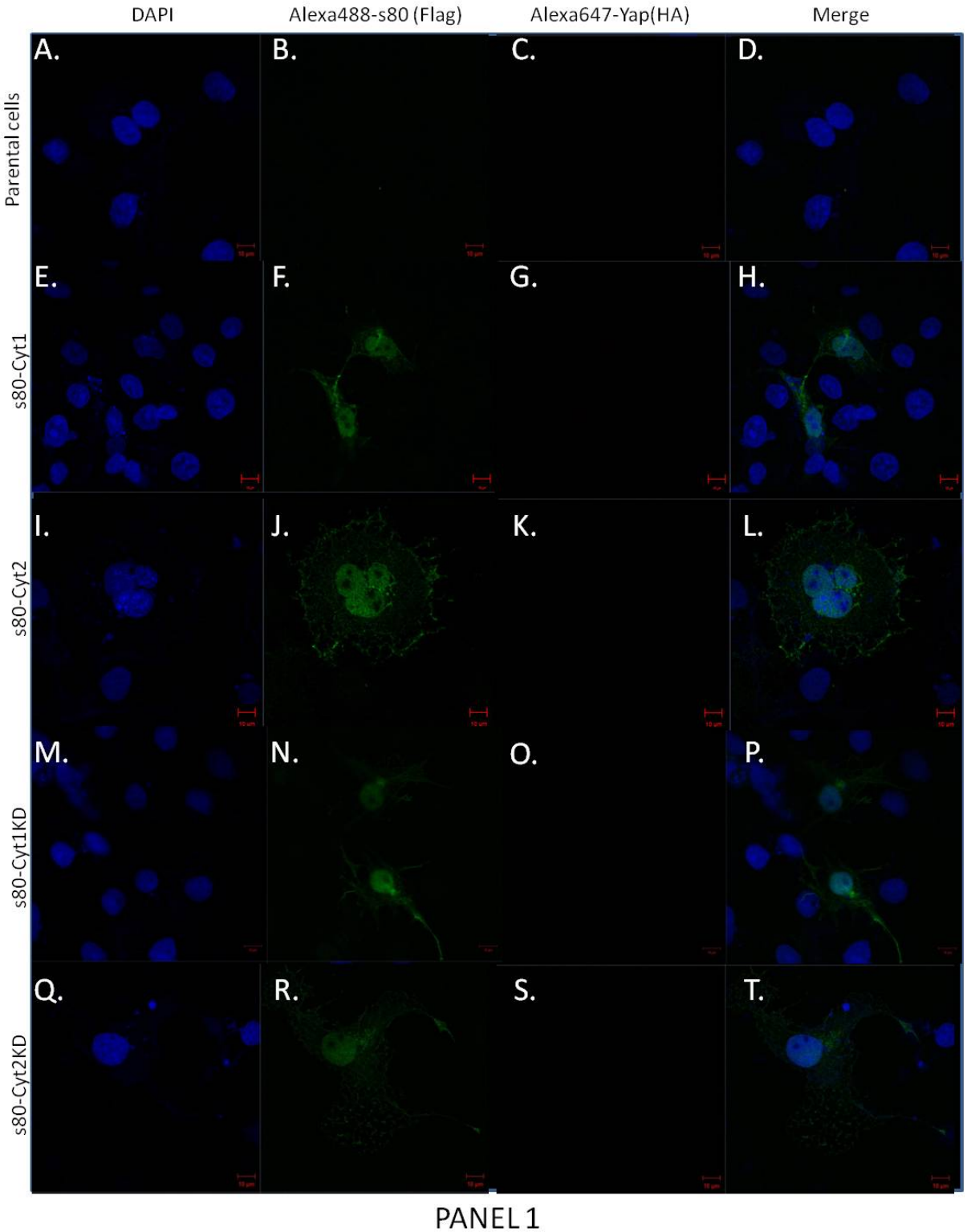
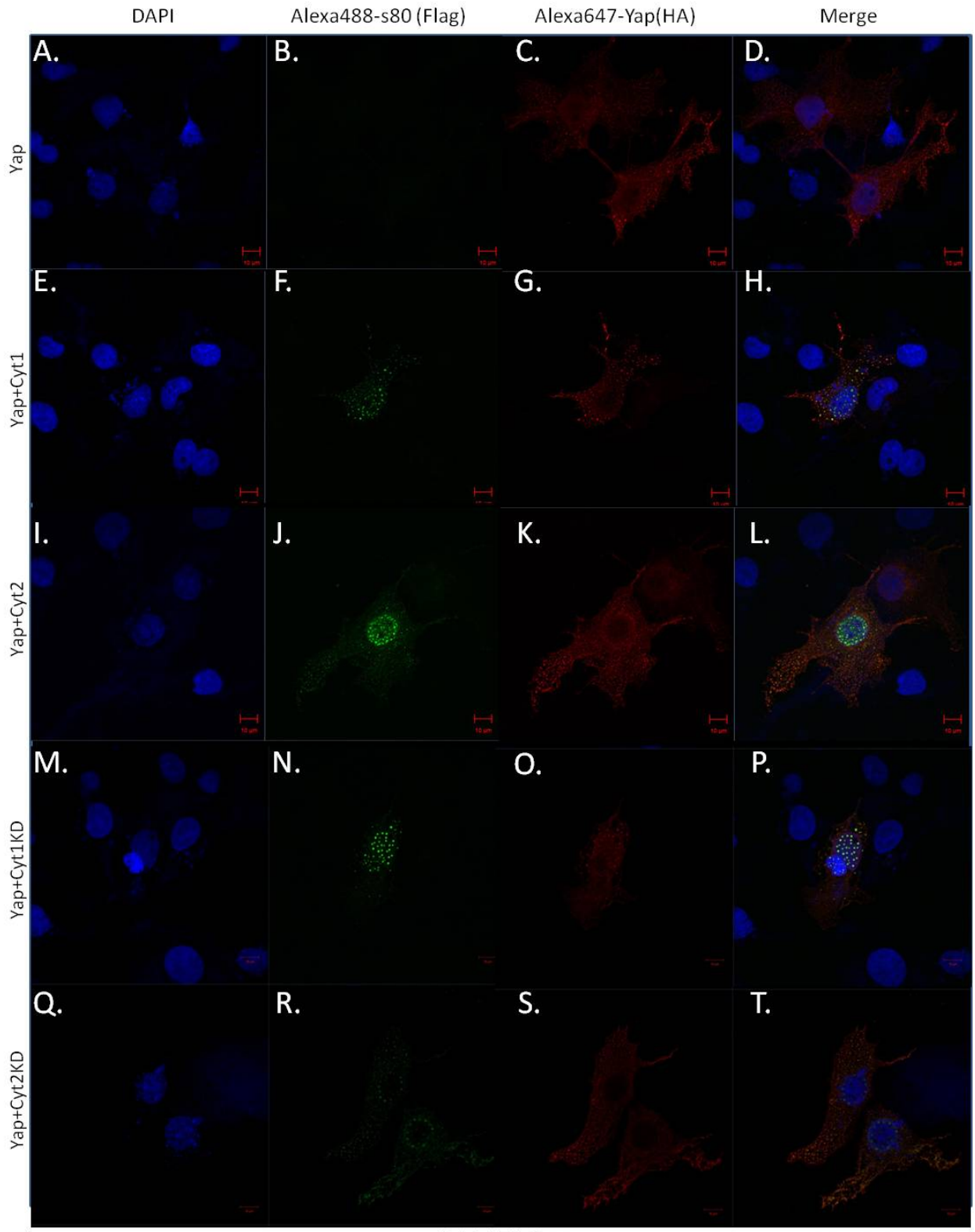


Figure 6. Expression levels of Yap and HER4-s80 in MCF7 cells. Expression levels were assayed 48h after transfection. No HER4-s80:Yap interaction or Yap tyrosine phosphorylation was detected.



PANEL 1

Figure 7. Localization of Yap and HER4-s80 isoforms in COS7 cells. COS7 cells were transfected with indicated constructs and 24h later plated on slides. After 24h to allow for cell attachment and recovery, cells were fixed, permeabilized and stained with anti-HA antibody coupled to Alexa647 and anti-Flag antibody coupled to Alexa488 (Cell Signaling). Slides were mounted with mounting media for fluorescence containing DAPI and analyzed with Zeiss LSM710 confocal microscope. All photographs taken at 63x with oil immersion. Scale bars represent 10 µm.


DAPI
Alexa488-s80 (Flag)
Alexa647-Yap(HA)
Merge
Yap
Yap+Cyt1
Yap+Cyt2
Yap+Cyt1KD
Yap+Cyt2KD
A.
B.
C.
D.
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PANEL 2

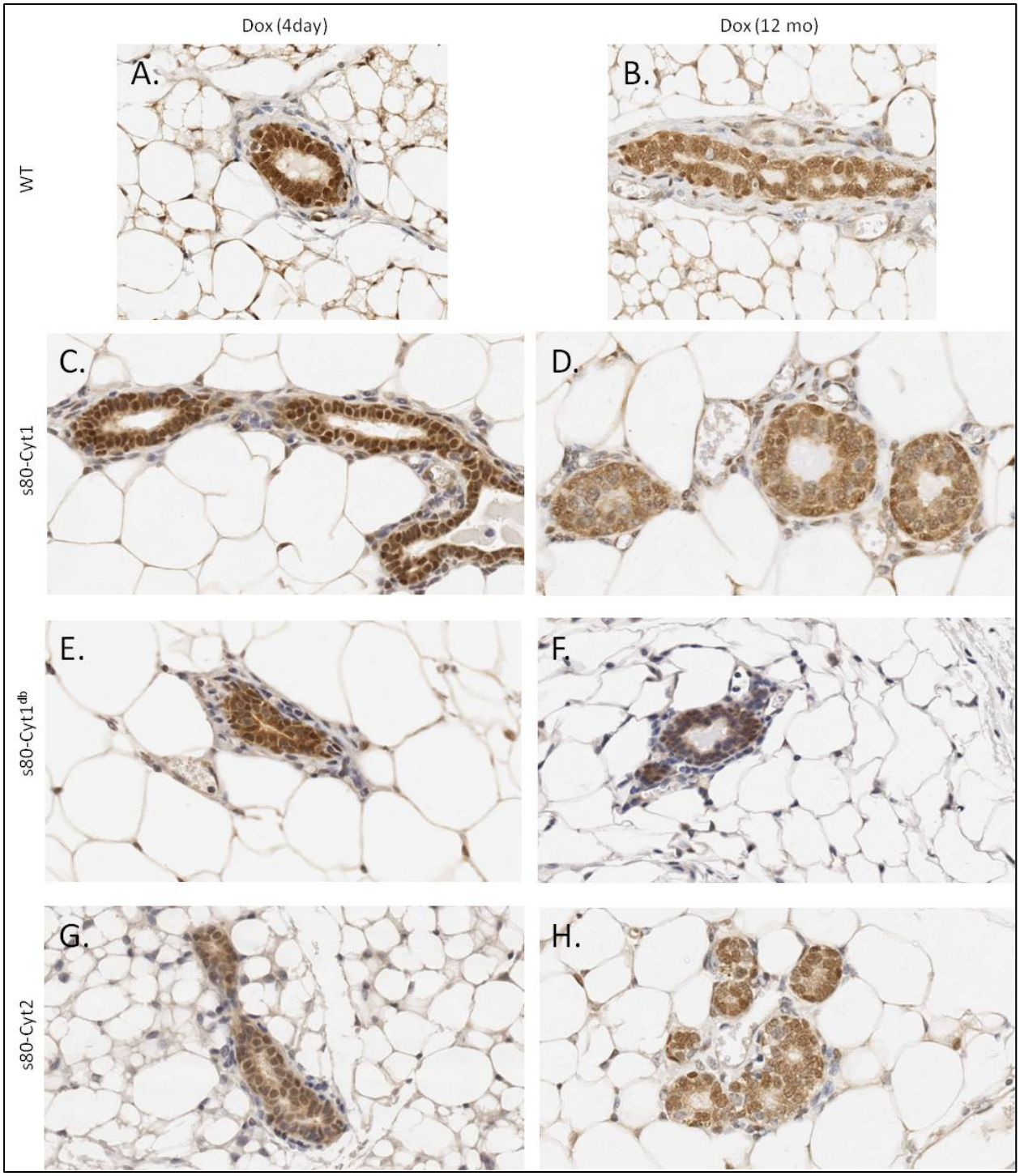

Figure 8. Localization of Yap in murine mammary epithelial cells. Mammary glands were harvested from WT and transgenic mice expressing human HER4-s80-Cyt1, -Cyt2 or degradation resistant –Cyt1^{db} for either 4 days or 12 months. Tissue was fixed in formalin and embedded in paraffin then stained with Yap antibody following an established immunohistochemistry protocol. Sections were counterstained with hematoxylin. Slides were photographed at 40x.

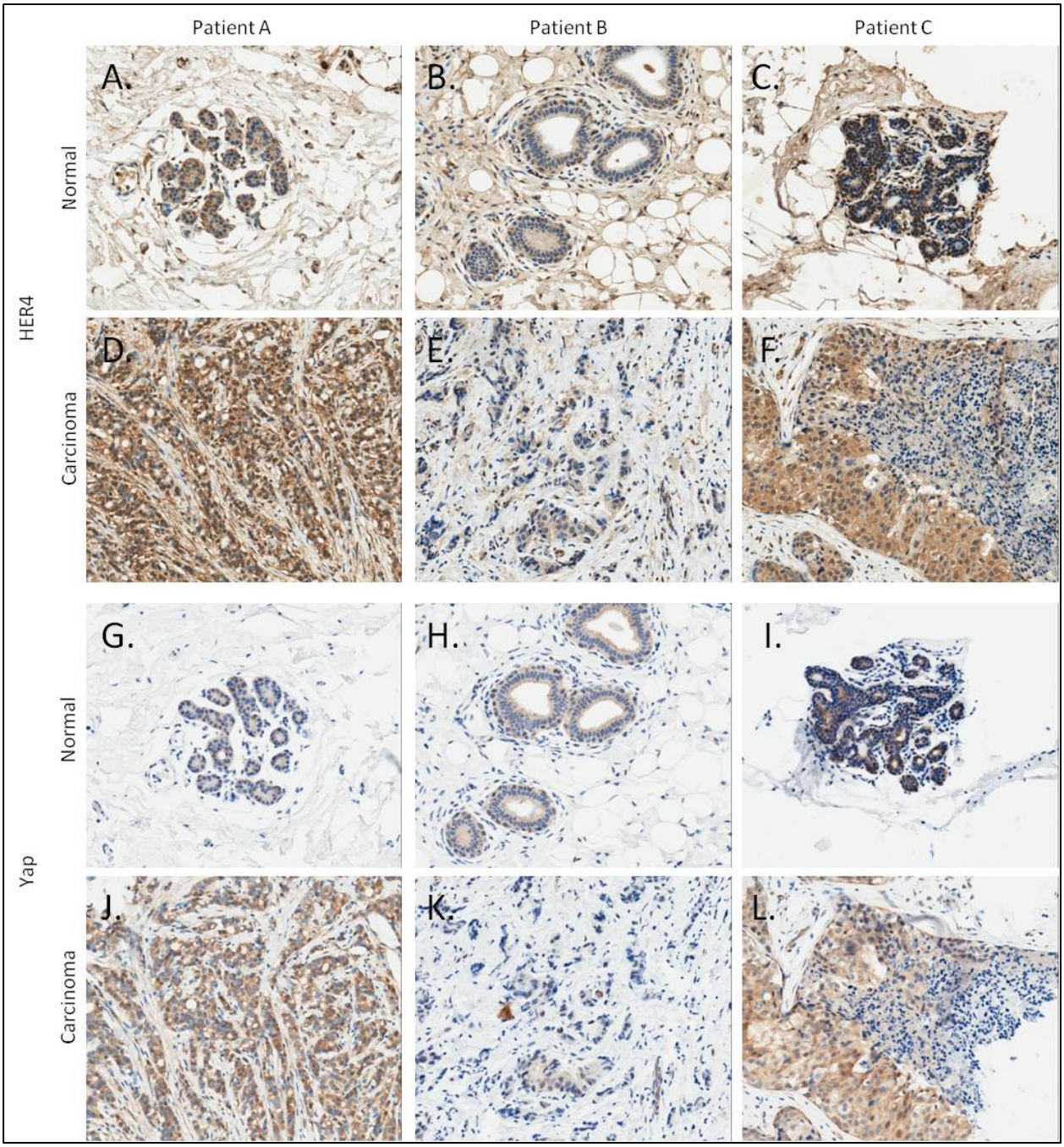

Figure 9. Yap and HER4 expression in human breast carcinoma and matching normal tissue. 10 samples of estrogen receptor-positive breast carcinoma and matching normal tissue were obtained from the University of North Carolina Tissue Procurement Facility. Sections were stained for Yap and HER4 following established immunohistochemistry protocol. Slides were scanned and scored for cytoplasmic and nuclear localization. Panels A-F: HER4 staining in normal (A-C) and carcinoma (D-F) samples from three patients. Panels G-L: Yap staining in normal (G-I) and carcinoma (J-L) samples from three patients. All images acquired at 20x.

Sequence	Modifications	Exp Value	Charge	First Scan	Last Scan	Ascore	Site Determining Ions	Localized
DSFFKPPEPKsHSRQAST	S11(Phosp)	0.001972287	3	4001	4001	5.99	1 / 22 @ 1 pks	no
DPFLNSGTyHSR	Y9(Phosp)	0.000189657	2	4038	4038	20.35	2 / 2 @ 8 pks	Yes
DPFLNSGTyHSR	Y9(Phosp)	0.001534511	3	4040	4040	6.93	2 / 8 @ 5 pks	no
DSFFKPPEPKSHSRQAsT	S17(Phosp)	0.000370655	3	4074	4074	0.06	0 / 1 @ 10 pks	no
DPFLNSGTYHsR	S11(Phosp)	0.000306881	2	4544	4544	57.75	4 / 4 @ 3 pks	Yes
DLGtLEG	T4(Phosp)	0.002079553	1	4551	4551	Only 1 site		
DDFLNsV	S6(Phosp)	0.001636703	1	6721	6721	Only 1 site		
DFLNsV	S5(Phosp)	0.00064561	1	6884	6884	Only 1 site		
DyLEAIPGTNV	Y2(Phosp)	0.000120773	1	7215	7215	0	0 / 0 @ 5 pks	no

Table 1. Mass spectrometry analysis of Yap1 phosphorylation sites. Yap1 was co-expressed with HER4-s80-Cyt1 in COS7 cells, lysate harvested 48h later and immunoprecipitated for HA-Yap and run on polyacrylamide gel. Band corresponding to HA-Yap (~72kDa), was excised and digested with AspN, then analyzed on LTQ Orbitrap mass spectrometer. ASCORE > 20 implies localization of phosphorylation on peptide [44]. These results were confirmed manually for peptide containing Y341 and Y394.

	Short Term (4 day)	**Long Term (one year)**
WT	**5**	**4**
s80-Cyt1^{db}	**3**	**3**
s80-Cyt1	**9**	**5**
s80-Cyt2	**7**	**3**

Table 2. Yap localization in murine mammary gland. Numbers of mice per each group for the analysis of Yap localization in mammary glands of transgenic mice expressing human HER4-s80-Cyt1, -Cyt1^{db} or –Cyt2.